Malonate Metabolism in Rat Brain Mitochondria†

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ABSTRACT: Rat brain mitochondria were found to have malonyl-CoA decarboxylase activity and a malonate-activating enzyme (malonyl-CoA synthetase). The decarboxylating enzyme had an apparent k_m of 0.5 mM and a pH optimum of 8.3. Methylmalonyl-CoA was an effective competitive inhibitor. Malonyl-CoA decarboxylase was tightly bound to mitochondria and could not be released by ultrasonication or by treatment with Triton X-100 (1%). The reaction product of malonyl-CoA decarboxylation, acetyl-CoA, was released into the incubation medium and not further oxidized by the Krebs cycle. Acetyl-CoA was hydrolyzed by acetyl-CoA hydrolase (EC 3.1.2.1) which was also associated with rat brain mitochondria. Malonyl-CoA decarboxylase from rat brain mitochondria differs from the decarboxylase in liver mitochondria which is localized in the matrix and which is readily released by ultrasonication. The liver enzyme has a pH optimum of 7.0. Rat brain mitochondria activated malonate to malonyl-CoA in the presence of ATP, CoA-SH, and Mg^{2+}. The enzyme had an apparent k_m of 0.08 mM and a pH optimum of 7.3. Malonyl-CoA synthetase was tightly bound to mitochondria or mitochondrial particles. Transfer of the CoA-SH moiety to malonate occurred also from succinyl-CoA and acetoacetyl-CoA. The reaction of acetoacetyl-CoA with malonate was probably catalyzed by succinyl-CoA:3-oxo-acid CoA-transferase (EC 2.8.3.5).

Demonstration of fatty acid biosynthesis in rat brain (Brady, 1960; Volpe and Kishimoto, 1972) and the requirement for malonyl-CoA in this process have focused attention on the origin and fate of this metabolite in the brain of mammals.

Initial investigations related to malonate metabolism in bacterial systems (Hayaishi, 1955; Nakada *et al.*, 1957) utilized labeled or unlabeled malonate as the substrate and documented the following pathway for degradation of malonate.

$$\text{malonate} + \text{ATP} + \text{CoA-SH} \rightleftharpoons \text{malonyl-CoA} + \text{ADP} + P_i \quad (1)$$

$$\text{malonyl-CoA} \rightarrow CO_2 + \text{acetyl-CoA} \quad (2)$$

Nakada *et al.* (1957) furthermore showed the conversion of acetyl-CoA evolving from reactions 1 and 2 to acetoacetate in rat liver when the Krebs cycle was inhibited by malonate at a concentration of 0.02 M. The further metabolism of acetyl-CoA was also supported by the fact that [2-^{14}C]malonate incubated with rat kidney slices gave rise to substantial amounts of labeled CO_2 from the oxidation of [2-^{14}C]acetyl-CoA which, in turn, resulted from the decarboxylation of the (unlabeled) C-3 of malonate. There was agreement among the investigators that activation of malonate had to precede decarboxylation and that ATP, CoA-SH, and magnesium were necessary for the reaction. Nakada *et al.* (1957) found the decarboxylating enzyme within mitochondria of rat kidney. This subcellular distribution of malonyl-CoA decarboxylase has been confirmed for several organs including the brain (Scholte, 1969; Landriscina *et al.* 1971). For rat liver mitochondria, a location within the mitochondrial matrix space was postulated by Scholte (1969) and assumed for rat brain by Landriscina *et al.* (1971).

Information on malonyl-CoA decarboxylase in brain is extremely sparse. Hayaishi (1955) failed to observe malonate decarboxylation by brain homogenates but Nakada *et al.* (1957) recorded modest enzyme activity in brain tissue slices. Others have noticed the presence of malonyl-CoA decarboxylase during studies of fatty acid biosynthesis in brain mitochondria (Boone and Wakil, 1970) and synaptosomes (Koeppen *et al.*, 1973). It thus appeared desirable to learn more about the activation of malonate and the subsequent decarboxylation of malonyl-CoA in brain mitochondria.

Decarboxylation of malonyl-CoA accompanies cytoplasmic fatty acid biosynthesis and fatty acid elongation in subcellular particles of brain. Reversal of the acetyl-CoA carboxylase reaction (acetyl-CoA:CO_2 ligase (ADP), EC 6.4.1.2) also results in the release of CO_2 from the C-3 position of the malonate moiety. In the investigation herein described, malonyl-CoA decarboxylation that is unrelated to either fatty acid biosynthesis or reversal of the acetyl-CoA carboxylase reaction has been studied.

Materials and Methods

Isolation of Mitochondria. Brains of four adult male albino rats were removed quickly after sacrifice of the animals by spinal fracture and homogenized in 10 volumes of 0.32 M sucrose. The suspension was centrifuged at 1000*g* for 10 min to remove nuclei and cell debris, and the supernatant fluid was collected. The pellet was washed twice with 0.32 M sucrose and the corresponding supernatants were combined. The total supernatant, approximately 60 ml, was layered on top of discontinuous sucrose gradient of 0.8 and 1.2 M sucrose. Centrifugation for 2 hr at 50,000*g* yielded a pellet which was collected from the bottom of the tube, mixed, and redispersed in 0.32 M sucrose. The mixture was again centrifuged in the discontinuous gradient of 0.8 and 1.2 M sucrose, and the pellet of this step was redispersed and adjusted to 4 ml with 0.32 M sucrose. Ultrastructurally, the final pellet consisted of mitochondria of variable size. The cristae were clearly preserved in most organelles though some disruption had occurred in an estimated 10% of the mitochondria. Synaptosomes were very infrequent, and plasma membranes including synaptic membranes were absent.

Incubations. Intact, ultrasonicated, or detergent-treated mitochondria were used. For ultrasonic disruption, 4 ml of a mitochondrial suspension in 0.32 M sucrose were treated with a Bronwill Biosonik IV ultrasonicator in bursts of 15 sec for a

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total of 2 min. The temperature was held near 0° by cooling the tubes in ice between bursts. Detergent treatment consisted of adding Triton X-100 to mitochondrial suspensions to a final concentration of 1%. The mixture was allowed to stand at 0° for 12 hr.

Incubations were performed in ordinary Warburg flasks. The total volume of the incubation mixture was 2.0 ml. The center well contained a narrow strip of filter paper soaked in 1 N KOH to trap evolving CO_2. Carbon dioxide evolution from the following substrates was examined: 1,3-[^{14}C]malonyl-CoA; 2-[^{14}C]malonyl-CoA; 1-[^{14}C]malonate; 2-[^{14}C]malonate; 3-[^{14}C]pyruvate; 2-[^{14}C]pyruvate. The detailed composition of the incubation mixtures is listed in the legends to the tables and figures. The reactions were carried out at 37° for up to 30 min and terminated at the appropriate interval by the addition of 0.5 ml of 35% perchloric acid from the side arm. Shaking was continued for 30 min. The alkaline strips were then removed from the center wells and placed into liquid scintillation vials. Commercial liquid scintillation fluid was added and radioactivity was determined in a Packard 3320 liquid scintillation spectrometer. After centrifuging the perchloric acid treated medium to remove protein, the clear supernatant fluid was collected and processed as described below.

In separate experiments, where it was desired to separate mitochondria from supernatant of the reaction mixture, they were chilled in ice and centrifuged at 100,000*g* for 30 min. Supernatant and pellets were collected and promptly treated with 0.5 ml of 35% perchloric acid.

Release of Enzymes by Ultrasonication and Detergent Treatment. Mitochondrial suspensions in 0.32 M sucrose treated by ultrasonication or by Triton X-100 were centrifuged at 100,000*g* for 30 min to obtain a pellet and a clear supernatant fluid. The supernatant fluid was recovered by aspiration and the pellet was reconstituted in 0.32M sucrose. Malonyl-CoA decarboxylase and malonyl-CoA synthetase were examined in both fractions.

Column Chromatography. Perchloric acid extracts were neutralized to pH 6.0 with 2 M K_2CO_3. The resulting $KClO_4$ was allowed to precipitate at 0° for 30 min and was then removed by centrifugation. Carrier amounts of malonyl-CoA and acetyl-CoA were added to the neutralized extract. The mixture was absorbed onto a 1 × 15 cm column of DEAE-cellulose previously equilibrated with 0.005 M HCl, collecting 4.5-ml fractions (total volume 600 ml). Absorption at 260 nm was monitored, and aliquots of the eluate were assayed for radioactivity.

Assays of Metabolites. Acetyl-CoA was determined in neutralized perchloric acid extracts by the enzymatic method described by Tubbs and Garland (1969). NADH was added to the incubation mixture to allow stoichiometric determination. The concentration of malonyl-CoA in the commercial preparation was determined with the hydroxamate method of Hestrin (1949). To standardize the technique, the potassium salt of malonic acid monoethyl ester was prepared according to Freund (1884) and purified by recrystallization from hot ethanol and washing with diethyl ether.

Acetoacetate and D-(−)-β-hydroxybutyrate were determined according to Williamson *et al.* (1967) with commercial D-(−)-β-hydroxybutyrate dehydrogenase. ADP and AMP were determined enzymatically employing lactate dehydrogenase, pyruvate kinase, and myokinase. The enzymatic assay of ATP utilized hexokinase and glucose-6-phosphate dehydrogenase. Protein was determined with a biuret procedure or by the technique of Lowry *et al.* (1951).

Thin-Layer Chromatography (tlc) of Acetic Acid. Aliquots of the perchloric acid extracts and column eluates were saturated with solid Na_2SO_4, and 0.01 ml of glacial acetic acid was introduced as a carrier; 5 ml of diethyl ether was added and the mixture was shaken continuously for 5 hr; 4 ml of the ether phase was then taken to dryness by a gentle stream of nitrogen in an ice bath. The residue was taken up in small volumes of ether and rapidly spotted on tlc plates of 100-μ thick microcrystalline cellulose. Before sample application the plates were sprayed with a 1% solution of morpholine in methanol and were allowed to dry at room temperature. The plates were developed in the morpholine-containing solvent system (solvent III) of Osteux *et al.* (1958). After drying at room temperature for 20 min, standard acetic acid bands were visualized with alkaline 0.05% Bromocresol Green solution in isopropyl alcohol. The corresponding sample bands were scraped off and examined for radioactivity.

Thin-Layer Chromatography of Hydroxamic Acids. Reactions were stopped by addition of 0.5 ml of alkaline hydroxylamine (Hestrin, 1949) whereupon malonic acid monoethyl ester and acetylthiocholine (1 μmol each) were introduced as carriers. After 1 min, the mixture was neutralized with 1 N HCl and lyophilized. The solid residue was extracted with acetone for 24 hr at 0° and an aliquot was chromatographed on cellulose tlc plates with diethyl ether–formic acid–water (7:2:1; v/v) which separated malonic acid (R_F 0.87), acethydroxamic acid (R_F 0.78), and malonomonohydroxamic acid (R_F 0.5). Standard compounds were visualized with an acidic $FeCl_3$ spray, and corresponding sample bands were examined for radioactivity.

Preparation of Methylmalonyl-CoA, Succinyl-CoA, and Acetoacetyl-CoA. Methylmalonyl monochloride was prepared by refluxing methylmalonic acid in ether with thionyl chloride as described by Overath *et al.* (1962), and the resulting acid chloride was reacted with thiophenol in excess pyridine. After the sulfhydryl reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2)[1] had ceased the mixture was acidified with dilute HCl. The methylmalonyl monothiophenyl ester was extracted with several volumes of diethyl ether. The ether phase was washed with water and 0.05 M HCl, and color was precipitated by activated charcoal. The concentration of the thioester was determined by the Hestrin (1949) method.Thiophenol released by hydroxylaminolysis was removed by shaking the assay tubes with diethyl ether. Coenyme A, dissolved in a small volume of 0.1 M $KHCO_3$, was reacted with an excess of the thiophenyl ester for 1 hr at room temperature. Ether was then added to the alkaline solution to extract free thiophenol. The aqueous phase was acidified by the addition of a cation exchange resin (Dowex 50 W, H^+ form) and further thiophenol and unreacted thiophenyl ester were extracted by several changes of ether. After removal of the resin by filtration, the mixture was adjusted to pH 6.0 with $KHCO_3$ and chromatographed on a column of DEAE-cellulose as described above. The tubes containing methylmalonyl-CoA were pooled, and their volume was reduced by lyophilization. Excess salt was removed by chromatography on an ion retardation resin (Bio-Rad AG 11 A8). Labeled 2-methyl[^{3}H]methylmalonyl-CoA or 2-methyl[^{14}C]-methylmalonyl-CoA were made by the same method with the respective labeled acids. Succinyl-CoA was prepared according to Simon and Shemin (1953). Acetoacetyl-CoA was synthesized from diketene and CoA-SH (Lynen *et al.*, 1958).

Assay for Acetyl-CoA Hydrolase (EC 3.1.2.1). The assay mixture contained, in a final volume of 2 ml, Tris buffer (pH 8.3), 0.05 M; NaCl, 0.05 M; KCl, 10 mM; $MgCl_2$, 1 mM; ace-

[1] Abbreviations used are: Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); LDH, lactic dehydrogenase.

TABLE I: $^{14}CO_2$ Evolution from ^{14}C-Labeled Substrates.[a]

Substrate	Substrate concn (mM)	pH	CO_2 from Labeled Carbon (nmol mg of protein^{-1} 30 min^{-1})		
			Intact Mitochondria	Ultrasonicated Mitochondria	Detergent-Treated Mitochondria
1,3-[^{14}C]Malonyl-CoA (5)	0.5	8.3	110.10	145.3	187.5
2-[^{14}C]Malonyl-CoA (2)	0.5	8.3	0.01	0	0
2-[^{14}C]Pyruvate (3)	1.0	8.3	4.85	0.72	0
3-[^{14}C]Pyruvate (3)	1.0	8.3	4.00	0.38	0
2-[^{14}C]Pyruvate + 0.5 mM "cold" malonyl-CoA (2)	1.0	8.3	5.00	0.70	0
1-[^{14}C]Malonate (5)	0.1	7.3	5.04	4.99	5.14
2-[^{14}C]Malonate (2)	0.1	7.3	0	0	0

[a] For malonyl-CoA and pyruvate, the incubation system contained Tris buffer, 0.05 M; KCl, 10 mM; $MgCl_2$, 1 mM; NaCl, 50 mM; sucrose, 0.32 M; mitochondrial protein, 1–3 mg in a total volume of 2 ml. For malonate, the incubation system contained sodium phosphate buffer, 0.05 M; KCl, 10 mM; $MgCl_2$, 1 mM; CoA-SH, 0.1 mM; ATP, 1 mM; sucrose, 0.32 M; mitochondrial protein, 1–3 mg in a total volume of 2 ml. Substrate concentrations and pH were as indicated. Number of experiments is in parentheses.

tyl-CoA, 0.5 mM; Nbs_2, 1 mM; detergent-treated mitochondria, 1–3 mg of protein. Release of CoA-SH was measured at 312 nm. This assay was not suitable for the simultaneous assay of malonyl-CoA decarboxylase and acetyl-CoA hydrolase because Nbs_2 at a concentration of 1 mM inhibited malonyl-CoA decarboxylase.

Sources of Chemicals. All labeled substrates were purchased from New England Nuclear, Boston, Mass. Acetyl-CoA, malonyl-CoA, propionyl-CoA, glutaryl-CoA, hexokinase, and glucose-6-phosphate dehydrogenase were from P-L Biochemicals, Milwaukee, Wis. Sigma, St. Louis, Mo., supplied LDH, pyruvate kinase, myokinase, D-(−)-β-hydroxybutyrate dehydrogenase, and β-hydroxyacyl-CoA dehydrogenase.

Results

Carbon Dioxide Evolution from ^{14}C-Labeled Substrates. Data on the evolution of CO_2 from labeled malonyl-CoA, pyruvate, and malonate are compiled in Table I. Disruption of the mitochondrial pellet by ultrasonication or treatment with Triton X-100 resulted in a considerable increase of CO_2 evolution from 1,3-[^{14}C]malonyl-CoA but had no effect when 2-[^{14}C]malonyl-CoA was the substrate. Whatever enzyme source was chosen, there was negligible formation of CO_2 from the C-2 carbon of either malonyl-CoA or free malonate. The strong evolution of labeled CO_2 from 1,3-[^{14}C]malonyl-CoA occurred only from the labeled C-3 carbon. The C-1 carbon would not be expected to undergo oxidation to CO_2 because of the failure of the C-2 carbon to produce labeled CO_2. Small amounts of pyruvate were oxidized to CO_2. The 2-[^{14}C]pyruvate and the 3-[^{14}C]pyruvate gave rise to labeled CO_2 *via* oxidation of 1-[^{14}C]acetyl-CoA and 2-[^{14}C]acetyl-CoA, respectively, when incubated with intact or ultrasonicated mitochondria. Unlabeled malonyl-CoA did not significantly dilute the radioactivity recovered in CO_2 arising from 2-[^{14}C]pyruvate or 3-[^{14}C]pyruvate so that two different pools of acetyl-CoA arose from the respective substrates malonyl-CoA and pyruvate. Failure of 2-[^{14}C]malonate to yield labeled CO_2 indicated that activation of malonate to malonyl-CoA was followed by decarboxylation at the same submitochondrial location and by the same reaction mechanism as described above for substrate malonyl-CoA.

Malonyl-CoA Decarboxylase. Carbon dioxide evolution was not linear with protein concentrations in excess of 2 mg or with time (Figure 1). Acetyl-CoA, determined spectrophotometrically, rose with increasing protein concentration and time, but did not reach the levels expected from the CO_2 values. Radioactive acetate was recovered from the reaction mixture and largely accounted for the difference between the determined acetyl-CoA values and the CO_2. Overall recoveries were 70–80%. No acetoacetyl-CoA, β-hydroxybutyryl-CoA, or the respective free acids could be detected by spectrophotometric enzymatic assay.

A chromatogram of the perchloric acid extract of the incubation mixture is shown in Figure 2. In addition to the unreacted substrate malonyl-CoA, the radioactive peaks were, in order of their elution, acetic acid, malonic acid, and acetyl-CoA.

A double reciprocal plot for malonyl-CoA decarboxylase is shown in Figure 3. The apparent k_m can be calculated as *ca.* 0.5 mM. Methylmalonyl-CoA proved to be an effective com-

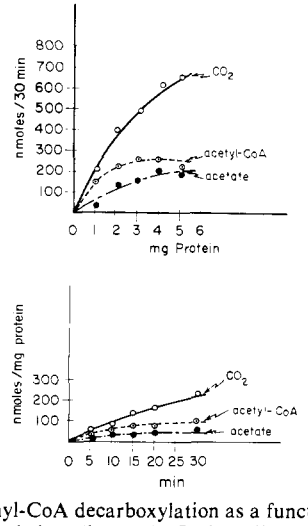

FIGURE 1: Malonyl-CoA decarboxylation as a function of protein concentration (top) and time (bottom). Carbon dioxide, acetyl-CoA, and acetate were measured as described under Materials and Methods. Enzyme source, detergent-treated mitochondria.

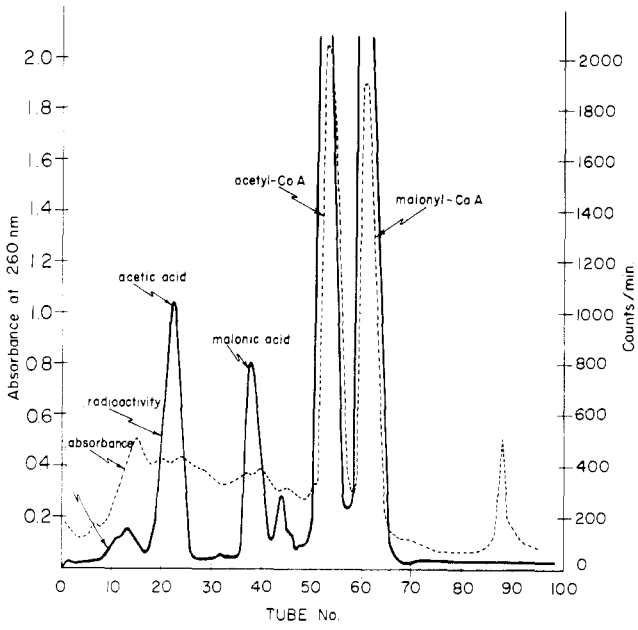

FIGURE 2: Malonyl-CoA decarboxylase. A perchloric acid extract of the incubation mixture after incubation with 1,3-[^{14}C]malonyl-CoA was neutralized and chromatographed on a column of DEAE-cellulose. Enzyme source, detergent-treated mitochondria.

petitive inhibitor of the enzyme (K_i = 0.05 mM). When 2-methyl[^{3}H]- or 2-methyl[^{14}C]methylmalonyl-CoA was used as inhibitor, there was no evidence of conversion to labeled succinate or propionate. The carboxylic acids were obtained by alkaline hydrolysis, acidification, ether extraction, and gas chromatography of the butyl esters. Other acyl-CoA had much higher K_i values (Table II) than methylmalonyl-CoA. Glutaryl-CoA was not inhibitory.

Malonyl-CoA decarboxylase was tightly bound to mitochondria since neither ultrasonication nor treatment with 1% Triton X-100 caused appreciable amounts to appear in the high-speed supernatant. After detergent treatment, approximately 5.4% of the total enzyme activity occurred in the particle-free supernatant, whereas 80% remained with the pellet (recovery 85.4%). After ultrasonication, only 1.5% of the enzyme activity occurred in the supernatant.

Malonyl-CoA Synthetase. When malonyl-CoA decarboxylase was simultaneously present, the evolution of CO_2 from free 1-[^{14}C]malonate was the most convenient way of assaying malonate activation in the presence of Mg, ATP, and CoA-SH. As illustrated in Figure 4, malonyl-CoA decarboxylase (pH optimum, 8.3) was still very active at the pH optimum of malonyl-CoA synthetase (7.3). Efforts to trap the biosynthesized mal-

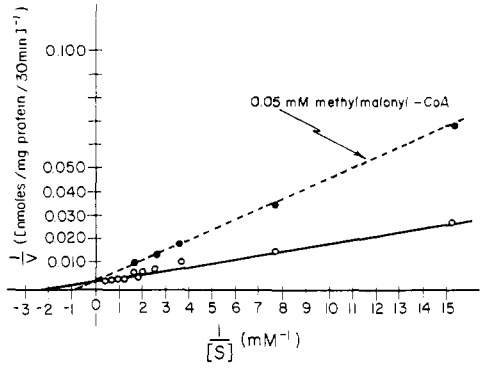

FIGURE 3: Malonyl-CoA decarboxylase. Double reciprocal plot.

TABLE II: Inhibitor Constants for Malonyl-CoA Decarboxylase.

Inhibitor	K_I (mM)
Acetyl-CoA	1.10
Propionyl-CoA	0.30
Methylmalonyl-CoA	0.05
Succinyl-CoA	0.58

onyl-CoA with neutral hydroxylamine required very high concentrations of the trapping reagent (0.2 M). At this concentration of hydroxylamine, the activating reaction was inhibited, confirming an earlier observation by Hayaishi (1955). Inhibition of malonyl-CoA decarboxylation by methylmalonyl-CoA was not suitable because of noncompetitive inhibition of malonyl-CoA synthetase. Since labeled acetyl-CoA appeared after incubation with 2-[^{14}C]malonate and no radioactivity was lost by decarboxylation of the C-3 carbon, the quantitative recovery of 2-[^{14}C]acethydroxamic acid would be a measure of malonate activation in the presence of malonyl-CoA decarboxylase. However, all enzyme sources also contained an active acetyl-CoA hydrolase which precluded the quantitative recovery of the label in acethydroxamate. As reported above, malonyl-CoA decarboxylase activity was low in the high-speed supernatant fractions of ultrasonicated or detergent-treated mitochondria. The malonyl-CoA synthetase assay of these supernatants was examined critically because of the requirement that decarboxylase be present for the evolution of $^{14}CO_2$ from 1-[^{14}C]malonate. The supernatant fluids always contained sufficient malonyl-CoA decarboxylase to completely decarboxylate malonyl-CoA resulting from the activation of malonate. Perchloric acid extracts did not contain unreacted labeled malonyl-CoA as determined by tlc of malonomonohydroxamic acid.

Malonate activation by mitochondrial enzyme preparations was linear with protein concentrations up to 5 mg and incubation times up to 30 min. The dependence of CO_2 evolution from 1-[^{14}C]malonate on CoA-SH and ATP concentrations is illustrated in Figure 5. In the absence of added CoA-SH, intact mitochondria decarboxylated malonate at about 40% of the rate achieved with a 0.5 mM concentration of added CoA-SH. In contrast, detergent-treated mitochondria had an absolute requirement for added CoA-SH, and higher concentrations of CoA-SH resulted in greater CO_2 evolution than could be achieved with intact mitochondria (Figure 5). At a CoA-SH concentration of 0.1 mM there was no difference in the enzymatic activity of intact and detergent-treated mitochondria. Ultrasonicated mitochondria behaved similarly.

In contrast to the variable CoA-SH dependence, all mitochondrial preparations required ATP (Figure 5).

Chromatography on DEAE-cellulose (Figure 6) showed that small radioactive peaks cochromatographed with both malonyl-CoA and acetyl-CoA. The peak preceding the large peak of unreacted malonic acid was identified as acetic acid.

The double reciprocal plot of malonyl-CoA synthetase established the apparent k_m at 0.08 mM malonate.

Ultrasonication released 5.6% of the total enzyme activity into the supernatant fluid; 85.4% remained with the pellet (recovery 91.0%). The values for the Triton X-100 treated mitochondria were: supernatant fluid, 4.2%; pellet, 41.3% (recovery 45.5%).

Activation of Malonate from Acyl-CoA. In separate experi-

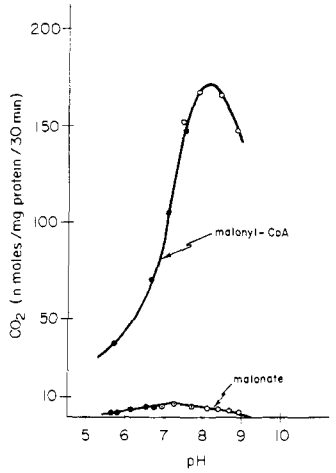

FIGURE 4: pH optima for malonyl-CoA decarboxylase and malonyl-CoA synthetase: enzyme source, detergent-treated mitochondria; (○) Tris buffer; (●) sodium phosphate buffer. No shifts occurred in the peak activity when intact or ultrasonicated mitochondria were used as the enzyme source instead of the Triton X-100 treated preparations.

ments, ATP and CoA-SH were replaced by acetyl-CoA, acetoacetyl-CoA, or succinyl-CoA (Table III). None of these could fully substitute for ATP and CoA-SH. Direct transfer of the CoA moiety from acetyl-CoA to malonate did not appear likely (Menon and Stern, 1960). Iodoacetamide was, therefore, used to inhibit acetoacetyl-CoA thiolase. The inhibitor strongly reduced CO_2 evolution from malonate in the presence of acetyl-CoA (Table III). The effect was not due to inhibition of malonyl-CoA decarboxylase, because iodoacetamide was not effective against this enzyme. This result confirms for brain mitochondria a previous observation by Lynen *et al.* (1962) on yeast malonyl-CoA decarboxylase. To further test the postulate that malonate activation in the presence of acetyl-CoA proceeds *via* formation of acetoacetyl-CoA and then by transfer of CoA-SH from acetoacetyl-CoA with concomitant release of acetoacetate, the following radiometric assay was devised. In one Warburg flask Triton X-100 treated mitochondria were incubated with 1-[^{14}C]malonate and unlabeled acetyl-CoA; a second flask contained the same incubation mixture except that the malonate was unlabeled and the acetyl-CoA was labeled (1-[^{14}C]acetyl-CoA). After termination of the reaction with perchloric acid and collection of labeled CO_2, the perchloric acid extract of the 1-[^{14}C]acetyl-CoA experiment was neutralized and chromatographed on a Dowex 1-X8 column as described by Von Korff (1969). Lithium acetoacetate (1 mg) was added as a carrier. Labeled acetoacetate from 1-[^{14}C]acetyl-CoA in the column effluent was compared to labeled CO_2 from 1-[^{14}C]malonate. Corrections were made for traces of labeled CO_2 evolving from 1-[^{14}C]acetyl-CoA and took into account also the fact that acetoacetate contained two labeled carbon atoms and that malonate activation yielded both 1-[^{14}C]malonyl-CoA and 3-[^{14}C]malonyl-CoA. As shown in Table III, equal amounts of CO_2 and acetoacetate were recovered. Since iodoacetamide inhibited the acetoacetyl-CoA thiolase reaction, both CO_2 evolution and acetoacetate formation were greatly reduced. Iodoacetamide, at a concentration of 5 mM, had no effect on the activation of malonate from acetoacetyl-CoA, but at 20 mM produced some inhibition of the transferase (Table III).

Origin of Labeled Acetate. When mitochondrial preparations were incubated with acetyl-CoA at pH 8.3 (the pH optimum of malonyl-CoA decarboxylase), CoA was released in linear fashion with time (up to 30 min) and with protein up to 5

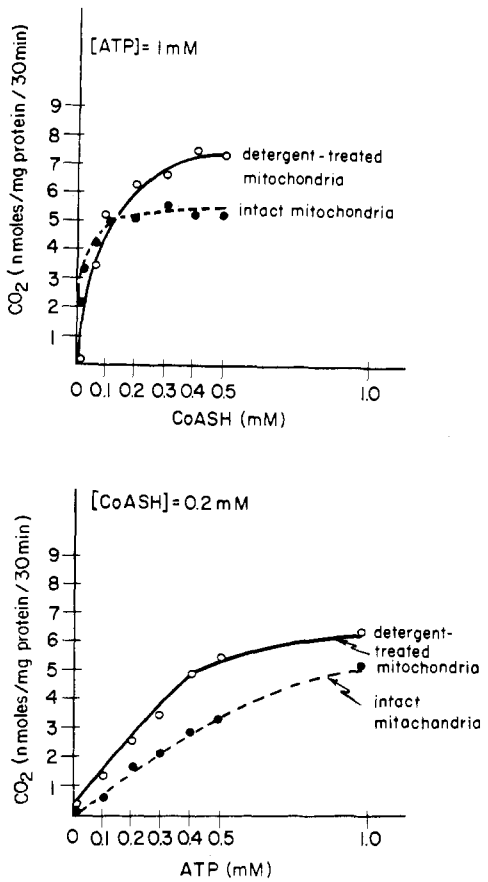

FIGURE 5: Malonyl-CoA synthetase. Carbon dioxide evolution as a function of CoA-SH (top) and ATP concentration (bottom).

mg. Eserine sulfate had no effect on the enzyme but diisopropyl fluorophosphate inhibited the enzyme of the detergent-treated preparations. The apparent k_m of acetyl-CoA hydrolase for the detergent-treated mitochondria was 28 μM acetyl-CoA at pH 8.3. Spontaneous hydrolysis of acetyl-CoA was negligible over a period of 30 min at this pH.

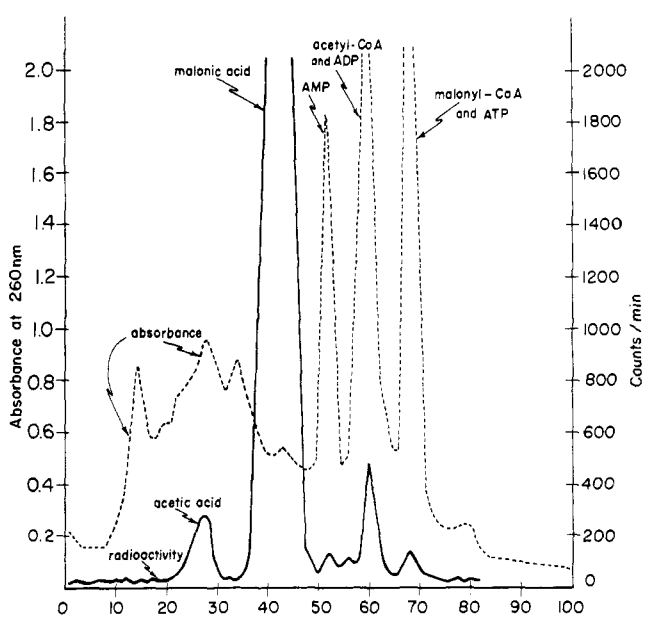

FIGURE 6: Malonyl-CoA synthetase. A perchloric acid extract of the incubation mixture after incubation with 1-[^{14}C]malonate was neutralized and chromatographed on DEAE-cellulose. Enzyme source, detergent-treated mitochondria.

TABLE III: Activation of Malonate from Acyl-CoA.[a]

Acyl-CoA	Concn (mM)	Addition or Deletion	CO_2 from Malonate nmol mg of protein^{-1} 30 min^{-1}	Acetoacetate from Acetyl-CoA
None			0.1	
None		+1 mM ATP + 0.1 mM CoA-SH	5.14[b]	
Acetyl-CoA	0.1		2.7	2.9
Acetyl-CoA	0.1	+5 mM iodoacetamide	0.9	Trace
Acetyl-CoA	0.1	+20 mM iodoacetamide	0.3	Trace
Acetyl-CoA	0.1	− malonate		Trace
Acetoacetyl-CoA	0.1		1.7	
Acetoacetyl-CoA	0.1	+5 mM iodoacetamide	2.5	
Acetoacetyl-CoA	0.1	+20 mM iodoacetamide	1.2	
Succinyl-CoA	0.1		1.9	

[a] The incubation system (final volume 2 ml) contained, in final concentration, sodium phosphate buffer (pH 7.3), 0.05 M; KCl, 100 mM; $MgCl_2$, 1 mM; malonate, 0.1 mM; acyl-CoA as indicated; mitochondrial protein, 1–3 mg. Triton X-100 treated mitochondria were used as the enzyme source. Results are averages of four experiments. [b] From Table I.

Discussion

Malonyl-CoA Decarboxylase. Rat brain mitochondria differed from mitochondria in other organs in their ability to decarboxylate malonyl-CoA. Landriscina *et al.* (1971) compared malonyl-CoA decarboxylation by mitochondria from rat liver, heart, kidney, and brain and found those from liver and kidney most effective. A pH optimum of 7.0 was shown for the liver enzyme and assumed to be the same for brain mitochondria. However, the pH optimum for the brain enzyme in this study was 8.3 (Figure 4). The apparent k_m for the liver enzyme was 42 μM (pH 7.0, 30°), whereas the respective value for brain was 500 μM (pH 8.3, 37°) (Figure 3). Ultrasonication of intact mitochondria from brain enhanced their activity (Table I) though the 3.8-fold increase reported by Landriscina *et al.* (1971) for a comparable mitochondrial fraction could not be confirmed. The matrix localization of the rat liver mitochondrial enzyme (Landriscina *et al.*, 1971; Scholte, 1969) did not obtain for rat brain. Only traces of the enzymatic activity could be recovered in the high-speed supernatant of ultrasonicated brain mitochondria, whereas 77.3% of the activity could be released from liver mitochondria. The 6.5-fold increase in enzyme activity in liver mitochondria after ultrasonication (Landriscina *et al.*, 1971) suggested that membrane barriers did not allow ready entry of the substrate into the matrix. In contrast, membrane barriers seemed of little significance in brain mitochondria (Table I), so that the submitochondrial localization of the cerebral enzyme is probably not the matrix. Precipitation with the pellet after such disruptive treatment as Triton X-100 supports binding to particles. The more accessible localization in brain mitochondria is also made likely by two other observations. (a) The reaction products, acetyl-CoA and acetate, were quantitatively recovered from the incubation medium. It is unlikely that such large amounts of acetyl-CoA can be released from the mitochondrial interior despite a 30-min incubation at 37° (Tuček, 1967). A matrix location of malonyl-CoA decarboxylase would require such a release of acetyl-CoA. (b) 2-[^{14}C]Malonyl-CoA did not give rise to $^{14}CO_2$ though some oxidation of 2-[^{14}C]acetyl-CoA by intact brain mitochondria would be expected in view of the small but definite pyruvate oxidation (Table I). The different fates of acetyl-CoA resulting from malonyl-CoA and pyruvate, respectively, were confirmed by the lack of influence of unlabeled malonyl-CoA on pyruvate oxidation (Table I). It should be recalled here that pyruvate alone and in the absence of ADP is a poor substrate for brain mitochondria (Stahl *et al.*, 1963; Clark and Nicklas, 1970; Von Korff *et al.*, 1971). The literature contains no previous reports on the fate of 2-[^{14}C]malonyl-CoA in mitochondria from any tissue. Data by Nakada *et al.* (1957) on the metabolism of 2-[^{14}C]malonate in liver and kidney slices indicate, however, that mitochondria from these sources may differ from those in the brain. In brain mitochondria enzymatic hydrolysis was the only established reaction for acetyl-CoA resulting from malonyl-CoA decarboxylation. Whole brain is known to have active acyl-CoA hydrolases (Srere *et al.*, 1959; Anderson and Erwin, 1971; Quraishi and Cook, 1972); 30% of the total acetyl-CoA hydrolase can be recovered in the brain mitochondrial fraction (Anderson and Erwin, 1971).

Malonyl-CoA Synthetase. In comparison to the decarboxylating enzyme, malonyl-CoA synthetase was much less active. The low CO_2 evolution from malonate by brain homogenates (Hayaishi, 1955) can be explained by the low activity of cerebral malonyl-CoA synthetase rather than by lack of decarboxylase. In a bacterial system, Hayaishi (1955) could replace ATP by acetyl phosphate and phosphotransacetylase. Acetyl-CoA resulting from the reaction of acetyl phosphate and CoA-SH appeared to transfer its CoA moiety to malonate and thus released free acetate. Menon and Stern (1960) showed for enzyme extracts of dog heart and skeletal muscle and for pig heart that the transfer of CoA-SH from acetyl-CoA to malonate, succinate, and glutarate was not direct but occurred only after formation of acetoacetyl-CoA. The favored substrate for the transfer of the CoA-SH group was succinate, but malonate activation occurred also though at $\frac{1}{50}$ of the rate. Transfer of the CoA-SH group to succinate and malonate appeared to occur by the same enzyme, *viz.*, succinyl-CoA:3-oxo-acid CoA-transferase (EC 2.8.3.5). Since this enzyme and acetoacetyl-CoA thiolase (acetyl-CoA:acetyl-CoA C-acetyltransferase; EC 2.3.1.9) are known to be present in adult rat brain (Williamson *et al.*, 1971), the data of Table III support the conclusion that activation of malonate by brain mitochondria proceeds by the mechanism described by Menon and Stern (1960).

The subcellular distribution of malonyl-CoA synthetase has not been studied in detail in mammalian tissues. The enzyme may not be exclusively mitochondrial. In rat brain mitochon-

dria it is closely associated with malonyl-CoA decarboxylase and thus may share its submitochondrial localization. The effectiveness of externally added CoA-SH on malonate activation by intact mitochondria suggests that no significant barriers are involved.

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A Calorimetric Study of the Thermal Transitions of Three Specific Transfer Ribonucleic Acids†

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ABSTRACT: Using differential heat capacity calorimetry, the thermal transitions of $tRNA^{Val}_{E.\ coli}$, $tRNA^{fMet}_{E.\ coli}$, and $tRNA^{Phe}_{yeast}$ have been examined, the latter one in greater detail due to the availability of larger amounts of pure material. The two species from *Escherichia coli* each show two well-separable thermal transitions in the calorimeter, a thermodynamically small transition at low temperature and a much larger one at elevated temperature. Neither of these transitions appear to be associated with changes in molecular weight. Only a single transition is seen for $tRNA^{Phe}_{yeast}$ and this is apparently the analog of the high-temperature transition seen for the other two species. Study of the $tRNA^{Phe}_{yeast}$ transition as a function of Mg^{2+} concentration reveals that there are very large increases in both the calorimetric enthalpy and heat capacity change as the transition temperature is raised by increasing the concentration of the divalent cation. The van't Hoff enthalpy changes have also been estimated and it is found that the ratio $\Delta H_{cal}/\Delta H_{VH}$ has a value of the order of 1.5. It is thereby concluded that the $tRNA^{Phe}_{yeast}$ thermal transition is not cooperative enough to be accurately approximated by an all-or-none (*i.e.*, two-state) model, but that it exhibits substantially more cooperativity than would be expected if the four cloverleaf helices melted independently. The results appear to be very consistent with the recently determined crystal structure of Kim *et al.* (*Science 179,* 285 (1973)), which reveals but two continuous helices with an indication of some interaction between them.

The thermal transitions of various tRNAs have been frequently investigated by spectrophotometric methods. The melting curves have generally (*e.g.*, Riesner *et. al.*, 1969) appeared to be biphasic or multiphasic in nature with the implication that different structural regions of the tRNA molecules melt out independently or semi-independently. In a particularly detailed investigation, Cole *et al.* (1972) studied the transitions of the Phe-, Val-, Tyr-, and fMet-tRNA from *Escherichia coli* and concluded that each of these show several different unfold-

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